

Retinoid Metabolism and Mode of Action

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Vitamin A and its derivatives (retinoids) are necessary for the maintenance of normal phenotypic expression. An attempt at understanding the biochemical role of vitamin A has led to the demonstration of a new pathway for retinol. In this pathway, vitamin A is phosphorylated to retinylphosphate (RP), which is then glycosylated to retinylphosphatemannose (MRP). These two derivatives have been found in a variety of tissues *in vivo* and *in vitro* and appear to be ubiquitous components of cellular membranes. The suggestion has been made that MRP may mediate specific cellular interactions by functioning as a lipid intermediate in the biosynthesis of specific glycoconjugates. A study on spontaneously-transformed mouse fibroblasts (Balb/c 3T12-3 cells) has shown that retinoids are active in increasing the adhesive properties of these cells as measured in an EDTA-mediated detachment assay. Various retinoids were tested for their activity in the adhesion test, and this activity was found to correlate well with their biological activity in maintaining the expression of normal epithelial differentiation in other systems. Retinoic acid, 5,6-epoxyretinol, and 5,6-epoxyretinoic acid were the most active compounds. Retinoids without biological activity in other systems were also inactive in inducing adhesive properties of 3T12-3 cells. Among these were the synthetic derivatives of retinol, anhydroretinol, and 4,5-monoeneperhydroretinol, and the phenyl derivative of retinoic acid, β -ionone, abscisic acid, and juvenile hormone, which are devoid of vitamin A activity in other systems, were also inactive in this system. Retinoid-induced changes in cell surface proteins were investigated but no difference in ^{125}I -fibronectin (MW 220,000) was detectable between retinoid-treated and untreated cells. However, these cells synthesized retinylphosphatemannose and the incorporation of 2- ^3H -mannose into a specific glycoprotein (gp 180) was found to be enhanced specifically by retinoid treatment. Investigations of the involvement of gp 180 in adhesion are in progress.

Introduction

Epidemiological studies indicate that the majority of human cancers are caused by exposure to environmental carcinogens (1). These can be inhaled, ingested with food, or absorbed through the skin and first come in contact with the epithelial surfaces of the body, which have a greater metabolizing activity than stromal fibroblasts. Related to this greater metabolic activity for chemical carcinogens is the fact that approximately 80% of human cancer originates in epithelial tissues (2).

Although exposure to carcinogens is essential for the neoplastic event to occur, host factors are very important modulators of the oncogenic response. These factors may be genetic or acquired. An ex-

ample of the former factors is the levels of carcinogen-activating enzymes, which appear to vary profoundly among different individuals (2). An example of acquired factors is vitamin A.

The interest in vitamin A and its derivatives, the retinoids (3), has derived from old and fundamental observations that the vitamin preserves the integrity of epithelial tissue (4). Feeding a vitamin A-deficient diet to hamsters causes replacement of the normal mucociliary epithelium of the trachea by squamous metaplastic and keratinized cells (3, 4). A similar response may also be caused by chemical carcinogens or mechanical injury. These conditions may be corrected by administration of vitamin A either to the whole animal or to the organ in culture (5, 6).

Because of these findings, a flurry of activity in the area of prevention of chemical carcinogenesis by retinoids has occurred in recent years with the scope of finding a potent and nontoxic retinoid, which might prevent neoplastic transformation in selected

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human populations at high risk of developing cancer.

Retinoids are also active in the alleviation of a variety of skin diseases, including actinic keratosis and basal cell carcinomas (7, 8).

Inasmuch as derivatives of retinoic acid (Fig. 1) are not stored in the liver and reach the target site in larger amounts than retinol, they have been used preferentially in preventive and therapeutic studies (3, 4, 7).

The proposed concept is that, by increasing the concentration of the retinoid in the target tissue, one enhances the potential of the tissue to maintain the normal phenotype, thereby counteracting the sequel of tissue alterations eventually leading to the neoplastic transformation, caused by carcinogens.

Although clearly such preventive studies do not require a full knowledge of the biochemical mechanisms of action of the retinoids, such knowledge may be crucial in the understanding of how the phenotypic expression of epithelial tissues is maintained.

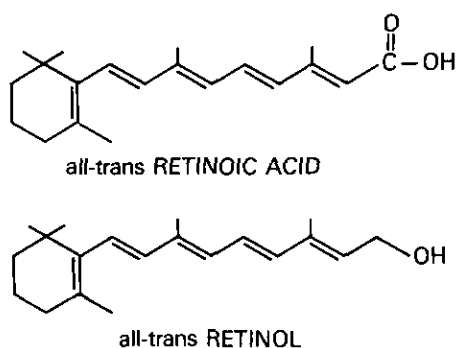


FIGURE 1. Structures of retinoic acid and retinol.

Results

Tracheal Epithelium

The integrity of the tracheal epithelium is lost in vitamin A deficiency. A study of the morphology of tracheal explants from vitamin A depleted hamsters and rats cultured in a vitamin A-depleted medium shows that the normally mucociliary epithelium is replaced by a squamous metaplastic epithelium, which keratinizes and sheds keratin into the medium (5, 6).

As in other epithelial tissues, a major biochemical defect in vitamin A deficiency in the respiratory mucosa occurs at the level of the biosynthesis of specific glycoproteins (9). Such alterations in glycoprotein biosynthesis may have important consequences for the membrane. The carbohydrate moieties of glycoproteins appear to act as "lectins,"

in that they bind specifically to sites which recognize their terminal sugar at the nonreducing end of the macromolecule (10).

Ashwell and Morrell and their collaborators (11) have shown that glycoproteins are readily removed from the blood stream of the rat, when their terminal sialic acid residues are removed and the penultimate sugar, galactose, is exposed to allow recognition by a molecule at the surface of the hepatocyte which sequesters the circulatory glycoprotein into the hepatocyte (11) and away from circulation. Similar recognition properties have been suggested by Roseman (12) to mediate intercellular interactions and the phenomenon of cellular adhesion.

There is now considerable evidence to indicate that vitamin A is directly involved in controlling the biosynthesis of specific glycoproteins in all epithelial tissues so far studied (9). Most of this work has been conducted in the whole animal, using radioactively labeled monosaccharides as precursors of glycoproteins. More recent work has confirmed these findings in tissue culture systems. Particularly, cultures of mouse epidermal cells have been shown to respond to retinyl palmitate by a remarkable (5- to 11-fold) increase in the incorporation of galactose, mannose, and glucosamine into epidermal glycoproteins (13).

Similar results were obtained in organ cultures of corneal epithelial cells from the eyes of rats. In this system it was shown that the corneal epithelium responds to culturing in the presence of retinol or retinoic acid by a selective increase in the incorporation of monosaccharides and without effect on the incorporation of leucine (14). The incorporation of glucosamine into high molecular weight glycoproteins (greater than 200,000 daltons) was specifically enhanced by vitamin A, either in organ culture or *in vivo* (14). Thus, vitamin A seems to control properties of biological membranes by controlling the biosynthesis of specific glycoproteins of the membrane.

Incorporation of ³H-Benzo(a)pyrene into Normal and Vitamin A-Deficient Hamster Tracheas

A faulty membrane may allow a greater amount of environmental pollutants inside the cell.

In a series of experiments we set out to measure the amount of ³H-benzo(a)pyrene, which was incorporated into negatively charged lipids of hamster tracheas from normal and vitamin A-depleted animals.

Tracheas (10 per group) were incubated in culture medium L-15 for 30 min at 37°C. The medium contained either 5 μ Ci of ³H-3,4-benzo(a)pyrene (spe-

cific radioactivity 11 Ci/mmmole), 5 μ Ci of 15- 3 H-retinol (specific radioactivity 1.25 μ Ci/mmmole) or 5 μ Ci of 14 C-mannose (50 μ Ci/mmmole). Retinol and mannose were used to study the specificity of the effect on benzo(a)pyrene. The incorporation of these radioactive precursors into membrane phospholipids was measured by chromatography of the lipidic extract (chloroform/methanol 2/1) on columns (0.75 \times 45 cm) of DEAE-cellulose acetate. The free precursors were eluted off the column with about 400 ml of 99% methanol. The radioactivity associated with negatively charged lipids was eluted with a gradient of 100 ml of 99% methanol to 100 ml of 100mM ammonium acetate.

Table 1 shows that the amount of each of the three precursors incorporated into negatively charged lipids of the tracheas from depleted hamsters was several fold greater than in normal tracheas.

The vitamin A-deficient tracheas incorporated 3.9 times as much benzo(a)pyrene into negatively charged lipids as their normal counterparts (Table 1). This finding is in agreement with previous results, which describe a larger amount of radioactive benzo(a)pyrene bound to the DNA of deficient tracheas (15). However, retinol and the monosaccharide mannose were also incorporated in larger amounts in the phospholipidic fraction of the deficient organs (Table 1), suggesting that general permeability characteristics of the deficient epithelium are profoundly altered in vitamin A deficiency. It is therefore reasonable to consider that the epithelial uptake of toxic substances from the environment may be modulated by the nutritional status of the animal.

Involvement of Retinoids in Adhesion

Vitamin A also affects the adhesive properties of cells. Glycoproteins, (16), glycolipids, and glycosyltransferases (12) have been implicated as the molecular determinants of adhesion. Inasmuch as retinol and retinoic acid are essential constituents of

membranes and modulators of the biosynthesis of specific glycoproteins, it was of interest to determine whether they could influence adhesion.

The system of choice was the spontaneously-transformed mouse fibroblasts (Balb/c 3T12-3 cells), because these cells display very poor adhesive properties in culture and can be lifted from the culture dish surface in an EDTA-mediated detachment assay (17). The cells appear round and display the typical morphology of transformed cells with very poor adhesion to each other (Fig. 2a). However, when cultured in the presence of 3.3×10^{-6} M retinol or retinoic acid, they acquire morphological characteristics of "normal" cells (Fig. 2b). Moreover, they remain attached to the plate, when treated with EDTA, under conditions which lift the untreated cells. Eventually, retinoid-treated cells also come off the plate, but only after prolonged EDTA treatment. Interestingly, saturation density was not affected markedly by retinoid treatment and the more adhesive, retinoid-treated cells had the same plating efficiency as the untreated 3T12 cells in dense (90%) and sparse culture conditions (20%).

Inasmuch as this assay might be useful to measure retinoid activity, the structure-activity relationship was investigated in this system. Retinol, retinoic acid, and their 5,6-epoxy derivatives were the most active compounds (18, 19). Derivatives of retinoic acid with activity in the hamster trachea differentiation system (3) were also active in increasing adhesion. Derivatives of retinol and retinoic acid, which were devoid of any vitamin A activity in other systems, were also inactive in increasing adhesion (18, 19). The free carboxyl group of retinoic acid was necessary for activity, since esterification or amide formation led to inactive compounds.

In conclusion, it appears that this newly found biological activity of retinoids in increasing adhesion of 3T12 cells correlates well with their vitamin A activity in other biological systems; it also offers an easily accessible and relatively fast assay for vitamin A activity.

An investigation of the biosynthesis of glycoproteins in this system of transformed cells is now under study. The incorporation of 2- 3 H-mannose into glycoprotein is greatly enhanced by retinoid treatment at the same time as the increase in adhesion is observed. Particularly the biosynthesis of a mannose-containing glycoprotein of molecular weight 180,000 daltons (gp 180) was found to be highly stimulated by retinoids, whereas not much effect was noticed on the biosynthesis or the iodination of the extracellular fibronectins, which have been implicated in the phenomenon of intercellular and cell to substrate adhesion (16).

In 1970 we first suggested that vitamin A controls

Table 1. Incorporation of 3 H-benzo(a)pyrene, 3 H-retinol, and 14 C-mannose into negatively charged lipids of normal and vitamin A-deficient hamster tracheas.^a

	Incorporation in 30 min at 37°C, μ mole/10 tracheas		
	Normal (N)	Deficient (D)	D/N
Benzo(a)pyrene	1.95×10^{-7}	7.66×10^{-7}	3.92
Retinol	16.9×10^{-7}	89.5×10^{-7}	5.29
Mannose	1.83×10^{-5}	5.09×10^{-5}	2.78

^aConditions of incubation and processing of the lipid extracts are described in the text.

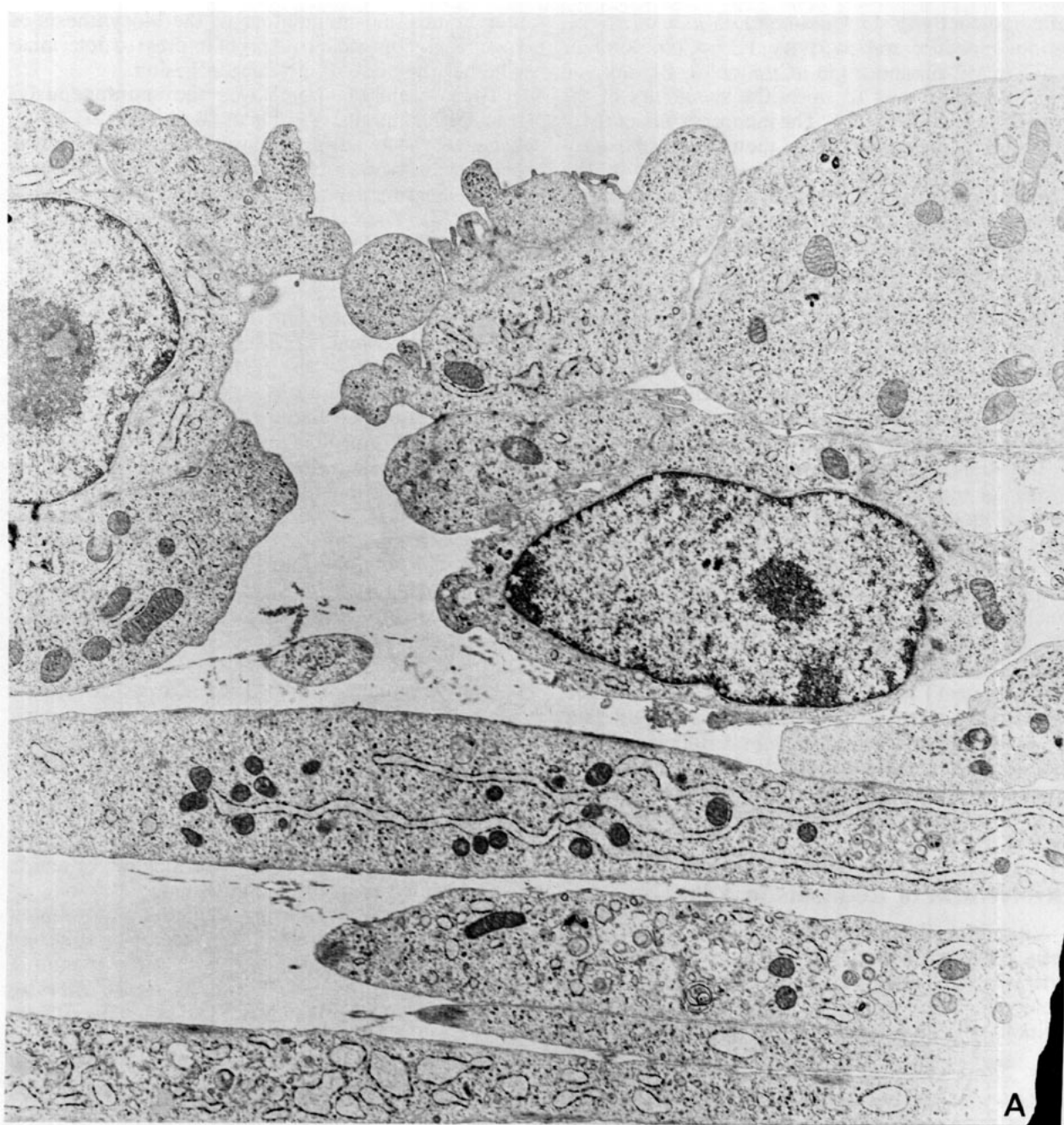


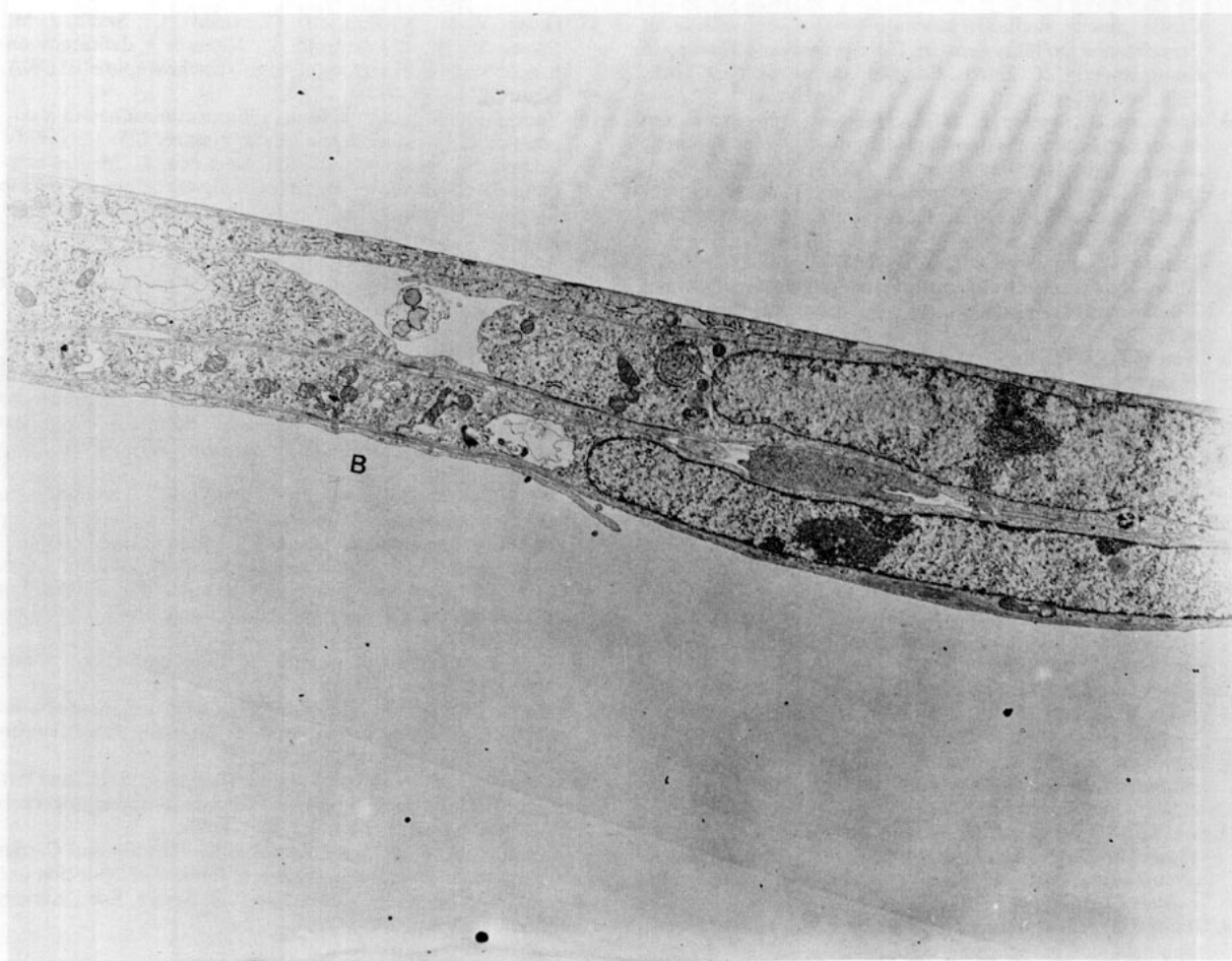
FIGURE 2. Transmission electron microscopy of (A) control and (B) retinoic acid-treated (B) 3T12 cells ($\times 3500$). Cells were seeded at $10,000/\text{cm}^2$ and grown in the presence or absence of $3.3 \times 10^{-6} M$ retinoic acid for 6 days.

membrane function by its molecular involvement in the biosynthesis of glycoproteins (9). We proposed that the phosphorylated form of vitamin A carries glycosyl residues across the membrane bilayer to make them available for glycosylation of glycoproteins. This hypothesis has received considerable attention after the demonstration that phosphorylated vitamin A (retinylphosphate) is a component of epi-

thelial membranes (9). The biosynthesis of retinylphosphate (9) has been demonstrated *in vivo* in rat liver and intestine (9, 20) and *in vitro* in cultures of rat intestinal cells (21) and mouse epidermis (13).

Conclusions

In addition to the role of vitamin A in the visual cycle as the chromophore of rhodopsin (22, 23), evi-



dence is now emerging for a new biochemical function for the vitamin as a carrier of mannosyl residues for the biosynthesis of glycoproteins (9). Such a carrier role appears to be essential for the biosynthesis of mannose-containing glycoproteins, as suggested by studies in deficiency and excess vitamin A. There is in fact a 79% reduction in the amount of mannose bound to glycoproteins of the hepatocyte membrane in vitamin A deficiency and a 610% increase in the incorporation of mannose in excess vitamin A.

It is thus suggested that, if such glycoproteins are necessary to regulate membrane functions, the diverse morphological and functional manifestations of the action of vitamin A [i.e., mucociliary function in respiratory epithelium (9), adhesive properties in fibroblasts (17-19), hormonal responses in the reproductive systems (24), etc.] depend on the particular tissue. Moreover, it appears that, in addition to retinol, a metabolite of retinoic acid undergoes the same fate of phosphorylation and glycosylation (9), thus functioning as vitamin A.

It is tempting to suggest that the function of the

binding proteins for retinol and retinoic acid is a protective one (25) against oxidation and metabolism, prior to insertion of the vitamin in the hydrophobic environment of the membrane bilayer, where it functions in the phosphorylated form.

Inasmuch as the vitamin maintains, and pollutants perturb the normal phenotype of the mucociliary epithelium, it would be of interest to study whether toxic agents such as ozone interfere with the vitamin A-dependent biochemical processes of epithelial tissues by destroying the retinol, retinyl esters, and retinyl phosphate present in these tissues. It would also be of interest to measure whether an increased intake of nutritional factors such as vitamin A would have a protective effect against the action of toxic substances in the respiratory epithelium, exposed to the toxic agent.

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